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(54) Title: RECOMBINANT ERYTHROPOIETIN / IMMUNOGLOBULIN FUSION PROTEINS

(57) Abstract

Production and use of fusion proteins comprising erythropoietin and immunoglobulin polypeptides. The erythropoietin fusion proteins have an increased in vivo half–like, as compared to wild type erythropoietin. The frequency with which it must be administered in patients in need thereof is reduced, compared to naturally–occurring erythropoietin.

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RECOMBINANT ERYTHROPOIETIN/IMMUNOGLOBULIN FUSION PROTEINS

BACKGROUND OF THE INVENTION

Erythropoietin (EPO) is a glycoprotein hormone involved with the growth and development of mature red blood cells from erythrocyte precursor cells. It is a 166 amino acid polypeptide that exists naturally as a monomer. (Lin, F-K., *et al.*, *Proc. Natl. Acad. Sci. USA* *82*:7580-7584 (1985)).

Several forms of anemia, including those associated with renal failure, HIV infection, blood loss and chronic disease can be treated with this hematopoietic growth factor. Erythropoietin is typically administered by intravenous or subcutaneous injection three times weekly at a dose of approximately 25-100 U/kg. One problem with erythropoietin therapy is that, although quite effective, this form of therapy is very expensive. Estimated costs for the treatment of chronic dialysis patients have ranged from $8,000-10,000 per patient per year.

Another problem encountered in the practice of medicine when using injectable pharmaceuticals is the frequency at which those injections must be made in order to maintain a therapeutic level of the compound in the circulation. For example, erythropoietin has a relatively short plasma half-life (Spivak, J.L., and Hogans, B.B., *Blood, 73*:90 (1989); McMahon, F.G., *et al.*, *Blood, 76*:1718(1990)), therefore, therapeutic plasma levels area rapidly lost, and repeated intravenous administrations must be made. An alternative route of administration is

subcutaneous injection. This route offers slower absorption from the site of administration, thus causing a sustained release effect. However, significantly lower plasma levels are achieved and, thus, a similar frequency of injection, as is required with intravenous administration, must be used to get a comparable therapeutic effect. It would be advantageous to have available an erythropoietin composition which has an extended circulating half-life to avoid such problems.

SUMMARY OF THE INVENTION

The present invention relates to fusion proteins comprising erythropoietin or erythropoietin-like molecules, and immunoglobulin polypeptide chains. The fusion proteins described herein are referred to as erythropoietin/immunoglobulin fusion proteins, or erythropoietin fusion proteins. Specifically, the erythropoietin fusion proteins of the present invention have increased biological activity wherein biological activity is defined as an increased *in vivo* half-life relative to wild type, or naturally-occurring, erythropoietin. The erythropoietin fusion proteins of the present invention can also exhibit increased biological activity as manifested by an increase in stimulation of hematopoiesis when compared to wildtype erythropoietin. Such biological activity can be independent of an increased *in vivo* half-life, or in addition to increased *in vivo* half-life. Because the erythropoietin/immunoglobulin fusion proteins of the present invention have increased biological activity, the frequency with which they must be administered is reduced from the frequency of administration of naturally-occurring erythropoietin.

Additionally, if erythropoietin fusion protein of the present invention is used, a reduced quantity of erythropoietin fusion protein over the course of treatment of an individual would be necessary than if naturally-occurring erythropoietin is used.

The immunoglobulin polypeptide chains of the present invention comprise the constant region of the immunoglobulin molecule. The chains can comprise either the heavy chain constant region or both the heavy and light chain constant regions, also referred to herein as immunoglobulin constant and light chain domains. The present invention also relates to multimers (also referred to herein as oligomers) of erythropoietin/immunoglobulin fusion proteins comprising two or more, e.g., four, erythropoietin/Ig fusion protein monomers, typically joined by interchain covalent linkages, such as disulfide bonds. These erythropoietin/Ig fusion proteins, referred to herein as dimers (if two monomers are joined), or tetrameres (if four monomers are joined), also exhibit the increased biological activity described herein. Also encompassed by the present invention is an erythropoietin/immunoglobuin fusion protein wherein an erythropoietin molecule replaces the V_L and V_H chains of the immunoglobulin.

In one embodiment of the present invention, DNA encoding erythropoietin, or an erythropoietin-like molecule, is fused at its N-terminus to DNA encoding an immunoglobulin constant region. In another embodiment of the present invention, DNA encoding erythropoietin, or an erythropoietin-like molecule, is fused at its C-terminus to the DNA encoding an immunoglobulin constant region. The fused sequence is expressed in competent cells, resulting

in production of an erythropoietin/immunoglobulin fusion protein that has biological activity.

In yet another embodiment of the present invention, the erythropoietin, or erythropoietin-like molecule, is modified to remove carbohydrate chains, or the erythropoietin, or erythropoietin-like molecule, is mutated to alter, or delete, naturally-occurring glycosylation sites from the molecule. DNA encoding non-glycosylated erythropoietin, or non-glycosylated erythropoietin-like molecule, is then fused to DNA encoding an immunoglobulin constant region.

Additionally, the DNA encoding erythropoietin can be mutated to increase biological activity (e.g., alanine 101 erythropoietin) and fused to DNA encoding an immunoglobulin constant region. DNA encoding an immunoglobulin constant region is typically modified to substantially decrease or eliminate Fc receptor binding or complement fixing activity.

Specifically encompassed by the present invention are the nucleic acids encoding the fusion proteins described herein and their encoded amino acid sequences.

The present invention further relates to methods of producing the erythropoietin/immunoglobulin fusion proteins with increased biological activity described herein, and to methods of their use, for example, in the treatment of anemia.

Thus, as presented herein, fusion protein compositions comprising erythropoietin and immunoglobulin, which exhibit increased biological activity relative to wild type erythropoietin, are now available. Moreover, the erythropoietin fusion proteins of the present invention can be dimerized, or tetramerized, with additional

erythropoietin fusion proteins to produce multimeric erythropoietin fusion proteins with prolonged *in vivo* half-life.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the production and use of erythropoietin/immunoglobulin fusion proteins with increased biological activity. The erythropoietin/immunoglobulin fusion proteins described herein are also referred to as erythropoietin/immunoglobulin chimeras, chimeric protein or hybrid proteins.

The erythropoietin/immunoglobulin fusion proteins of the present invention comprise polypeptides having two components: erythropoietin, or an erythropoietin-like molecule, (defined herein as an erythropoietin fragment, analog, variant, mutant or derivative of erythropoietin), and the constant region of an immunoglobulin, or a fragment, analog, variant, mutant or derivative of the immunoglobulin. The fusion protein can also comprise one or more additional components, such as signal or targeting sequences.

Typically, the first component of the fusion protein is erythropoietin, which is a protein hormone involved with the growth and development of red blood cells from erythrocyte precursor cells. Erythropoietin is produced in the kidney in response to hypoxia (e.g., red blood cell loss due to anemia) and regulates red blood cell growth and differentiation through interaction with its cognate cellular receptor. Wild type, or naturally-occurring, erythropoietin is defined herein to include recombinant erythropoietin (Jacobs, K., *et al.*, *Nature*, 313:806-813 (1985)), the teachings of which are herein incorporated by

reference), or naturally-occurring erythropoietin which has been isolated and purified from blood (Miyake, T., *et al.*, *J. Biol. Chem.*, *252*:5558-5564 (1977)) or sheep plasma (Goldwasser, E., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, *68*:697-698 (1971)), or chemically synthesized erythropoietin which can be produced using techniques well-known to those of skill in the art. Erythropoietin is a 166 amino acid polypeptide that exists naturally as a monomer. The predicted secondary structure of erythropoietin has been reported (McDonald, J.D., *et al.*, *Mol. Cell. Biol.*, *6*:842-848 (1986)).

As defined herein, the term "erythropoietin" also encompasses biologically active fragments of erythropoietin, erythropoietin mutants (also referred to herein as muteins) and erythropoietin analogs, variants and derivatives, referred to herein as erythropoietin-like molecules.

As used herein, the biological activity of erythropoietin is defined as the ability to regulate red blood cell growth and differentiation through interaction with its cognate cellular receptor, or the antigenic property of inducing a specific immunological response as determined using well-known laboratory techniques. For example, a biologically active, or functionally active, fragment of erythropoietin can induce an immunological response which produces antibodies specific for erythropoietin (anti-erythropoietin antibodies).

To be "functionally" or "biologically active" an erythropoietin-like molecule typically shares substantial sequence (amino acid) similarity (e.g., at least about 65%, typically at least about 80% and most typically about 90-95%) with the corresponding sequences of wild type, or

naturally-occurring, erythropoietin and possesses one or more of the functions of wild type erythropoietin thereof.

The erythropoietin of the present invention is understood to specifically include erythropoietin polypeptides having amino acid sequences analogous to the sequence of the wild type erythropoietin. Such proteins are defined herein as erythropoietin analogs. An "analog" is defined herein to mean an amino acid sequence with sufficient similarity to the amino acid sequence of wild type erythropoietin to possess the biological activity of the protein. For example, an analog of a polypeptide can contain "silent" changes in the nucleic acid sequence which encodes a polypeptide, wherein one or more amino acid residues differ from the amino acid sequence of wild type erythropoietin, yet possesses, e.g., the ability to stimulate red blood cell production or maturation. Examples of such differences include additions, deletions or substitutions of residues.

Also encompassed by the present invention are proteins that exhibit greater or lesser biological activity of wild type erythropoietin, such as described in U.S. Patent No. 5,614,184, the teachings of which are incorporated herein by reference. Exemplary mutant erythropoietin molecules with either increased or decreased biological activity as described in U.S. Patent No. 5,614,184 include, for example, alanine 101 erythropoietin; alanine 103 erythropoietin; alanine 104 erythropoietin; alanine 105 erythropoietin and alanine 108 erythropoietin.

The present invention also encompasses biologically active fragments of erythropoietin. Such fragments can include only a part of the full-length amino acid sequence of erythropoietin yet possess biological activity. As used

herein, a "biologically active fragment" means a fragment that can exert a biological or physical effect of the full-length protein, or has a biological characteristic, e.g., antigenicity, of the full-length protein. For example, a biologically active erythropoietin fragment can induce the growth and differentiation of red blood cells or stimulate an immunogenic response. The antigenicity of a peptide fragment can be determined, for example, as described in Geysen, *et al.*, WO 84/03564, the teachings of which are herein incorporated by reference. Such activities and characteristics are described above. Such fragments can be produced by amino and carboxyl terminal deletions as well as internal deletions. Also included are active fragments of the protein as obtained by enzymatic digestion. Such peptide fragments can be tested for biological activity as described herein.

"Derivatives" and "variants" of erythropoietin are erythropoietin proteins that have been modified. They include erythropoietin polypeptides that have been modified by alterations in their amino acid sequence. They also include truncated and hybrid forms of erythropoietin. "Truncated" forms are shorter versions of erythropoietin, for example, with amino terminal, or carboxyl terminal residues removed.

Variants can be produced using methods discussed below. The erythropoietin gene can be mutated *in vitro* or *in vivo* using techniques well known to those of skill in the art, for example, site-specific mutagenesis and oligonucleotide mutagenesis. (See for example, U.S. Patent NO. 5,614,184.) Manipulations of erythropoietin can be made at the protein level as well. Any of numerous chemical modifications can be carried out by known

techniques including, but not limited to, specific chemical cleavage by cyanogen bromide, trypsin and papain.

In general, mutations can be conservative or non-conservative amino acid substitutions, amino acid insertions or amino acid deletions. More preferably, DNA encoding an erythropoietin amino acid sequence variant is prepared by site-directed mutagenesis of DNA that encodes a variant or a nonvariant version of erythropoietin. Site-directed (site-specific) mutagenesis allows the production of erythropoietin variants through the use of specific oligonucleotide sequences that encode the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the techniques of site-specific mutagenesis are well known in the art, as exemplified by publications such as Edelman *et al.*, *DNA* 2, 183 (1983). The site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al.*, *Third Cleveland Symposium on Macromolecules and Recombinant DNA*, A. Walton, ed., Elsevier, Amsterdam (1981). This and other phage vectors are commercially available and their use is well-known to those skilled in the art. A versatile and efficient procedure for the construction of oligonucleotide directed site-specific mutations in DNA fragments using M13-derived vectors was published by

Zoller, M.J. and Smith, M., *Nucleic Acids Res.* 10:6487-6500 (1982)). Plasmid vectors that contain a single-stranded phage origin of replication can also be employed to obtain single-stranded DNA. Veira *et al.*, *Meth. Enzymol.* 153:3 (1987). Alternatively, nucleotide substitutions can be introduced by synthesizing the appropriate DNA fragment *in vitro*, and amplifying it by PCR procedures known in the art.

In general, site-specific mutagenesis can be performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea, *et al.*, *Proc. Natl. Acad. Sci. USA* 75, 5765 (1978). This primer can then be annealed with the single-stranded protein sequence-containing vector, and subjected to DNA-polymerizing enzymes such as E. coli polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector can then be used to transform appropriate host cells such as JM101 cells, and clones can be selected that include recombinant vectors bearing the mutated sequence arrangement. Thereafter, the mutated region can be removed and placed in an appropriate expression vector for protein production.

The PCR technique can also be used in creating amino acid sequence variants of an erythropoietin polypeptide. When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence

from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers can be designed to overlap the position of the mutation and to contain the mutation. The sequence of the other primer is preferably identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 500 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair as described above results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product can be used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more) part ligation.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al. Gene* 34, 315 (1985). The starting material can be

the plasmid (or vector) comprising the erythropoietin DNA to be mutated. The codon(s) within the erythropoietin to be mutated are identified. There must be unique restriction endonuclease sites on each side of the identified mutation site(s). If such restriction sites do not exist, they can be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the erythropoietin DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated erythropoietin DNA sequence, that can be expressed to produce mutated erythropoietin.

Naturally-occurring erythropoietin is glycosylated at three N-linked positions and one O-linked position. (Powell, J.S., *et al.*, *J. Biol. Chem.*, 263:17516-17521 (1988)). Although studies indicate that glycosylation is important to *in vivo* biological activity, non-glycosylated erythropoietin has been shown to have *in vitro* activity which is even increased over glycosylated erythropoietin activity. However, the half-life of non-glycosylated erythropoietin is extremely short. (Yamaguchi, K., *et al.*, *J. Biol. Chem.*, 266:20434-20439 (1991)). Encompassed by

the present invention are fusion proteins comprising non-glycosylated erythropoietin, or non-glycosylated erythropoietin-like molecules, and an immunoglobulin constant region polypeptide. Non-glycosylated erythropoietin is defined herein as erythropoietin lacking N-linked and/or O-linked carbohydrates (oligosaccharide chains). Site-directed mutagenesis can be used to alter or eliminate the carbohydrate attachment sites or erythropoietin, for example, as described in Dube, S., *et al., J. Biol. Chem., 263*:17516-17521 (1988). Alternatively, carbohydrates can be enzymatically removed from erythropoietin, using techniques well-known to those of skill in the art, such as N-glycanase digestion. Immunoglobulins are stable plasma proteins with substantial half-lives. An immunoglobulin constant region polypeptide, or a fragment, mutant, analog or variant thereof, fused to non-glycosylated erythropoietin can reasonably extend the plasma half-life of non-glycosylated erythropoietin, e.g., a plasma half-life of at least two times greater than the half life of the non-fused, non-glycosylated erythropoietin.

The second component of the fusion protein of the present invention is the constant region of an immunoglobulin. The erythropoietin molecule can be fused to the immunoglobulin constant domain at either its C-terminus or its N-terminus. In one embodiment, the C-terminus of erythropoietin is fused to the N-terminus of an immunoglobulin constant region polypeptide, (e.g. N-Epo-C→N-Ig-C, where the erythropoietin molecule is located upstream of the Ig constant region). In another embodiment, the C-terminus of the immunoglobuin constant region is fused to the N-terminus of erythropoietin (e.g.,

N-Ig-C→N-Epo-C, where the erythropoietin molecule is located downstream of the Ig constant region). Specifically encompassed by the present invention are biologically active fragments, analogs, mutants, variants and derivatives of immunoglobulin. The terms "fragments, analogs, mutants, variants and derivatives" are described above. As defined herein, the biological activity of an immunoglobulin fragment, analog, mutant, variant or derivative is the ability of the immunoglobulin fragment, analog, mutant, variant or derivative to extend the *in vivo* half-life of the fused erythropoietin molecule, or to enhance the potency of the fused erythropoietin. The enhanced potency of erythropoietin results from, e.g., the use of oligomerized rather than monomeric erythropoietin. Use of oligomerized erythropoietin can stimulate the activity of the erythropoietin receptor, which must be oligomerized to induce intracellular or signaling, resulting in growth and differentiation of red blood cells. In this case, the fusion protein acts as an erythropoietin agonist.

Immunoglobulin and biologically active fragments, analogs, mutants, variants and derivatives thereof, are well-known to those of skill in the art. The classes of immunoglobulin are IgG, IgD, IgA, IgE and IgM. The IgG class is subdivided into subclasses IgG1, IgG2, IgG3 and IgG4. These subclasses are further sub-divided, e.g., IgG2a. IgA is divided into subclasses IgA1 and IgA2. Immunoglobulin comprises heavy and light polypeptide chains. The Fc portion of the immunoglobulin is defined as a naturally-occurring or synthetically produced polypeptide homologous to the immunoglobulin C-terminal domain that is produced upon papain digestion.

The constant region of the heavy chains of all mammalian immunoglobulin exhibit extensive amino acid sequence similarity. The constant region domains are termed CH1, CH2 and CH3. A "hinge" region joins CH1 to CH2-CH3. The hinge region typically comprises the polypeptide homologous to the naturally-occurring immunoglobulin region which includes the cysteine residues at which disulfide bonds form which link the two heavy chains of the immunoglobulin. Representative sequences of hinge regions for human and mouse immunoglobulin can be found in ANTIBODY ENGINEERING, A PRACTICAL GUIDE, Borrebaeck, C. A. K., ed., W. H. Freeman and Co., 1992, the teachings of which are herein incorporated by reference.

In the present invention, the entire immunoglobulin heavy chain constant region (CH1-hinge-CH2-CH3) can be fused to the erythropoietin molecule. Alternatively, the immunoglobulin constant region can comprise all, or a portion of the hinge region, the CH2 domain and the CH3 domain. The immunoglobulin constant region can also comprise the CL1 domain of an immunoglobulin light chain.

If the immunoglobulin heavy chain polypeptide is used to construct the erythropoietin fusion protein, the polypeptide can be altered, or mutated, to eliminate, or significantly decrease complement fixation activity and/or Fc receptor binding. For example, the amino acid residues which comprise the Fc binding site, or the C1q binding site, of the immunoglobulin constant region amino acid sequence can be deleted or mutated, thereby eliminating, or substantially decreasing Fc binding or C1q binding.

The fusion proteins of the present invention are typically produced by recombinant methods well-known to those of skill in the art. (See e.g., Morrison, S.L.,

Science, 229:1202-1207 (1985); Mark, M.R., *et al.*, *J. Biol. Chem.*, 267:26166-26171 (1992); Peppel, K., *et al.*, *J. Exp. Med.*, 174:1483-1489 (1991)). For example, a nucleic acid (either DNA or RNA) construct can be produced, comprising a nucleic acid sequence encoding erythropoietin, or a fragment, mutant, variant or analog thereof, joined to a nucleic acid sequence encoding the hinge region, CH2 and CH3 domains of an immunoglobulin constant region. Nucleic acid sequences encoding immunoglobulin are known to those of skill in the art. Such sequences can be obtained from cDNA libraries or synthetically produced. The erythropoietin/Ig nucleic acid construct can be introduced into an expression vector by methods well-known to those of skill in the art and introduced into a competent cell (e.g., a cell that is compatible with the vector sequences and capable of expressing the encoded fusion protein) which then expresses the erythropoietin/immunoglobulin fusion protein.

As another example, cDNA for V_L can be excised and cDNA sequence encoding erythropoietin, or a fragment, analog, mutant, variant or derivative thereof, is placed upstream of the constant region of an immunoglobulin light chain. An erythropoietin/Fcγ2a sequence and an erythropoietin CL1 sequence are then co-transfected into a competent mammalian cell, resulting in the production of a tetrameric erythropoietin/Ig fusion protein.

Alternatively, the amino acid sequence comprising the fusion protein can be synthesized by methods well-known to those of skill in the art.

The erythropoietin molecule can be joined to the Ig sequence at either the N-terminus or C-terminus of erythropoietin. At either terminus, the precise site of

joining the nucleic acid sequence encoding erythropoietin to the nucleic acid sequence encoding the immunoglobulin constant region can be determined by one of skill in the art by routine experimentation. Such sites are known. However, the nucleic acid sequence encoding erythropoietin and the nucleic acid sequence encoding the immunoglobulin constant region must be in the correct translational reading frame. The erythropoietin nucleic acid sequence can be joined directly to, e.g., the hinge region nucleic acid sequence, or, alternatively, additional nucleotides encoding a flexible protein sequence, (e.g., about 1 to about 20 amino acids) can be inserted prior to the hinge region nucleic acid sequence, as long as the inserted nucleotides do not interfere with the biological activity of the expressed fusion protein or do not confer any undesired activities, e.g., such as antigenity.

Nucleic acid sequences encompassed by the present invention include isolated and/or recombinant nucleic acid sequences which encode the erythropoietin/Ig fusion protein. Also encompassed by the present invention are nucleic acid sequences (DNA or RNA) which are substantially complementary to the sequences encoding the erythropoietin/Ig fusion proteins and nucleic acid sequences which hybridize with the erythropoietin/Ig fusion protein nucleic acid sequences. Hybridization conditions sufficient to identify nucleic acid sequences with substantial sequence identity to the erythropoietin/Ig fusion proteins of the present invention are known to those of skill in the art. Such conditions are described in *Current Protocols in Molecular Biology*, Ausubel, F.M., *et al.* eds. (1991), the teachings of which are incorporated herein by reference. Factors such as sequence length, base

composition, temperature and ionic strength affect hybridization. One of skill in the art can empirically determine hybridizing conditions to find substantially complementary sequences. As defined herein, substantially complementary means that the sequence need not reflect the exact sequence of the erythropoietin/Ig fusion protein, but must be sufficiently similar in identity to hybridize with the sequences of the present invention. For example, non-complementary bases, or additional bases can be interspersed in the sequences provided that the sequence has sufficient complementary bases to hybridize therewith.

The expressed erythropoietin/immunoglobulin fusion proteins are secreted from cells transformed with the DNA construct into the cell culture medium. The secreted fusion protein can be secreted as a single chain polypeptide, also referred to herein as a monomer. However, the fusion protein can associate via covalent or noncovalent (e.g., disulfide or hydrophobic) interactions to form multimeric fusion proteins, such as dimers or tertramers, which are also secreted into the culture medium. For example, the immunoglobulin constant region contains cysteine amino acid residues. Two fusion protein monomers containing cysteine residues can form interchain disulfide bonds, resulting in fusion protein dimers. These secreted fusion proteins can be recovered form the culture medium using techniques well-known to those of skill in the art.

To obtain recombinantly produced glycosylated erythropoietin/immunoglobulin fusion proteins, the preferred host cells are eukaryotic cells, e.g., mammalian cells such as CHO or COS. The fusion protein can contain glycosylation sites that are substantially the same as naturally-occurring erythropoietin and immunoglobulin, or

the glycosylation of the fusion protein can vary from that of the naturally-occurring proteins. For example, if the fusion proteins are produced in yeast or insect cells, by methods known to those of skill in the art, the glycosylation pattern can vary from the pattern of glycosylation obtained when the fusion protein is produced in mammalian cells.

Erythropoietin has a relatively short plasma half-life (Spivak, J.L. and Hogans, B.B., *Blood*, 73:90-99 (1989); McMahon, F.G., *et al.*, *Blood*, 76:1718-1722 (1990). Therefore, therapeutic plasma levels are rapidly lost, and repeated intravenous or subcutaneous administrations must be made to maintain therapeutic levels of erythropoietin. The erythropoietin/immunoglobulin fusion proteins of the present invention reasonably have an increased circulating *in-vivo* half-life. The increase in circulating half-life is extended by a factor of two or more when compared to naturally-occurring erythropoietin. For example, the *in vivo* half-life can be extended from minutes (as is typical with naturally-occurring erythropoietin) to hours or even days, with the fusion proteins described herein. Circulating half-life of the fusion proteins of the present invention can be tested using methods known to those of skill in the art. For example, the erythropoietin/immunoglobulin fusion protein can be injected into rabbits, and blood samples analyzed at specific time points. The biological activity of the erythropoietin component circulating as fusion protein present in the blood samples can be assayed by known methods, such as the Krystal bioassay (Krystal, G. *Exp. Hematol.*, 11:649-660 (1993), the teachings of which are herein incorporated by reference. Alternatively,

structural integrity of the erythropoietin fusion protein can be measured by reactivity with erythropoietin-specific antibodies such as described in Sytkowski, A.J. and Fisher, J.W., *J. Biol. Chem.*, 260:14727-14731, the teachings of which are herein incorporated by reference. The prolonged circulating half-life of the erythropoietin fusion protein is reasonably due to its increased size relative to monomeric erythropoietin, which would hinder its excretion from the body through the kidney, and reduce clearance by other mechanisms.

As a result of the work described herein, erythropoietin/immunoglobulin fusion proteins are provided which exhibit increased biological activity associated with prolonged *in vivo* half-life, or increased potency. This increase in biological activity indicates that an effective amount of erythropoietin/immunoglobulin fusion protein is substantially less than a comparable effective amount of naturally-occurring erythropoietin. The effective amount of erythropoietin is defined herein as the amount of erythropoietin required to elicit an erythropoietic response, as indicated by increased growth and/or differentiation of erythrocytic precursor cells. For example, the effective amount of erythropoietin fusion protein described herein, with a prolonged circulating half-life, will require less frequent administration than an equivalent amount of naturally-occurring erythropoietin. Thus, a reduced quantity of erythropoietin, administered as erythropoietin/Ig fusion protein, will be required over the course of treatment than is necessary if wild type erythropoietin is used.

The erythropoietin/immunoglobulin fusion protein with increased biological activity described herein can be used

in place of wild type erythropoietin whenever treatment with erythropoietin is called for. Specifically, the EPO/Ig fusion protein is used to treat an individual with a hematopoietic disease or deficiency. For example, erythropoietin fusion protein can be used for treatment in an individual experiencing anemia associated with renal failure, chronic disease, HIV infection, blood loss or cancer.

Erythropoietin is generally administered to humans. Effective treatment with erythropoietin requires maintaining a therapeutic blood level. This can be done by continuous administration, that is, by continuous intravenous injections, by discreet intravenous injections, or by subcutaneous injection. The erythropoietin/Ig fusion protein of this invention can be employed in admixture with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral administration that do not deleteriously react with the active derivatives, referred to herein as pharmaceutical compositions.

Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatine, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethy-cellulose, polyvinyl pyrrolidone, etc. For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, foams, or implants, including suppositories.

It will be appreciated that the actual preferred amounts of active compound in a specific case will vary according to the specific compound being utilized, the particular compositions formulated, the mode of application, the particular situs of application, and the individual being treated. Dosages for a given recipient will be determined on the basis of individual characteristics, such as body size, weight, age, gender and the type and severity of the condition being treated.

In addition, the erythropoietin fusion protein of the present invention, with increased biological activity, can be used in any *in vitro* application in place of wild type erythropoietin. For example, modified erythropoietin can be used in studies of erythropoietin receptor activity. It will again be appreciated that the amount of erythropoietin fusion protein with increased biological activity needed to achieve desired results, (e.g., increased hemoglobinization of red blood cell precursor cells) will be less than the amount of wild type erythropoietin required to achieve those desired results.

The present invention will now be illustrated by the following example, which is not intended to be limiting in any way.

EXEMPLIFICATION: PRODUCTION OF AN ERYTHROPOIETIN/ IMMUNOGLOBULIN FUSION PROTEIN

An erythropoietin/immunoglobulin fusion protein can be produced according to the methods described in Steurer, W., *et al.*, *J. Immunol.*, *155*:1165-1175 (1995), the teachings of which are incorporated, in their entirety, by reference. A brief description of a method of producing an Epo/Fcγ2a heavy chain chimeric fusion protein follows. Because of a

mutation of the Epo/Fcγ2a and C'1q binding sites of the Epo/Fcγ2a portion of the fusion protein, the fusion protein is expected to bind to its receptor, but not target antigen presenting cells (APCs) for lysis through antibody-cellular cytotoxicy (ADCC) or complement-directed cytolysis (CDC).

MATERIALS AND METHODS:

MONOCLONAL ANTIBODIES

The following monoclonal antibodies are used: rat anti-mouse IgG2a (PharmMingen, San Diego, CA, rat anti-mouse Ig2a-horseradish peroxidase (PharMingen), FITC-labeled goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO), biotinylated rabbit anti-rat mAb (Vector, Burlingame, CA), FITC-rabbit anti-hamster IgG (Pierce, Rockford, IL), and mouse IgG2a (k) and IgG3 (k) hybridoma proteins (Cappel, West Chester, PA).

CELL LINES

The following cell lines are used: murine IgG2a-secreting hybridoma 116-13.1 (American Type Culture Collection (ATCC), Rockville, MD), CHO-K1 (ATCC). CHO cells transfected with human FcγRI cDNA, and transfected CHO cells are described in Steurer, W., *et al.*, *J. immunol.*, *155*:1165-1175 (1995), and can be produced using standard laboratory techniques.

CELL CULTURE

Cell culture regions, unless otherwise stated are obtained from Life Technologies, Inc. (Grand Island, NY). Cells are grown in complete RPMI 1640, i.e., RPMI supplemented with L-glutamine, 10% heat-inactivated FCS, 10 mM HEPES, 0.1mM non-essential amino acids, 1 mM sodium

pyruvate; 5 X 10^{-5} M 2-ME (Sigma Chemical Co.); 100 U/ml penicillian; and 100 µg/ml streptomycin. CHO-K1 transfectants are maintained in DMEM with 5% FCS, 100 U/ml penicillian, and 100 mg/ml streptomycin. Transfected cell lines are cultured in Ultraculture (Bio-Whittaker, Walkersville, MD) serum-free media supplemented with L-glutamine, penicillin, and streptomycin.

PLASMIDS

The eukaryotic expression vector Rc/CMV (Invitrogen, San Diego, CA), is modified by deletion of all three BamH1 sites and its unique Apal site. The PCT II vector (Invitrogen) is used for TA cloning of cDNA amplified by the PCR.

GENETIC CONSTRUCTS

Total RNA is purified on a cesium chloride (Life Technologies, Inc.) gradient, from the murine IgG2a-secreting hybridoma 116-13.1 and then reverse cDNA using oligo-dT 12-18 (Pharmacia, Piscataway, NJ) primers and M-MLV reverse transcriptase (Life Technologies Inc.). The region of the Fcγ2a cDNA encoding the hinge, CH2 and CH3 domains of the heavy chain is then amplified by PCR using oligonucleotides designed to append unique *BamHI and* Xbal restriction sites onto the 5' and 3' enzymes (New England Biolabs, Beverly, MA) and gel purified in preparation for ligation (see below). A cDNA encoding the erythropoietin is amplified by PCR using oligonucleotides designed to append unique *Not*I and *BamHI* restriction sites onto the 5' and 3' ends of this cDNA PCR respectively. The cDNA is then cloned into the PCR II vector, excised using *Not*I (New England Biolabs) and *BamHI* restriction endonucleases, and

gel purified. Subsequently, the erythropoietin cDNA, the previously prepared Fcγ2a cDNA, and the cDNA of the modified Rc/CMV vector opened at the cloning site with *Not*I and *X*bal restriction endonucleases are mixed and ligated using T4 DNA ligase (Life Technologies, Inc.). The correct reading frame at the junction of the erythropoietin to Fc CDNAs is confirmed by DNA sequencing.

PCR-assisted, site-directed mutagenesis of the Fcγ2a cassette is used to render nonfunctional (a) the high affinity FcγRI receptor binding site by substituting Glu for Leu 235, localization of the binding site for the human high-affinity Fc receptor on IgG. (Duncan, A.R., *et al.*, *Nature* 332:563 (1988)) and (b) the C'1q binding site by substituting Glu 318, Lys 322 with Ala residues, the binding site for C1q on IgG (Duncan, A.R., and Winter, G., *Nature* 332:738 (1988)). The cDNA mutations are confirmed by Dna sequencing. Subsequence expression of these constructs results in erythropoietin/Fc fusion proteins with or without ADCC and CDC activity, respectively.

EPO/Ig EXPRESSION AND PURIFICATION

To achieve stable expression, 20μg of the murine EPO/Ig plasmid construct is linearized by *Pvu* I digestion (New England Biolabs) and electroporated into 10^7 CHO-K1 cells. Transformed CHO-K1 cells are selected with 1 mg/ml G418 (Life Technologies, Inc.), and subsequently cloned by limiting dilution. Established cell lines are then screened for EPO/Ig production by an ELISA specific for murine IgG2a. High producing clones are cultured in serum-free media for 12 days. Supernatant is size (0.2 μm pore) filtered, and Tris, pH 8.0 is added to a final concentration of 50 mM, and then passed over a protein A-

Sepharose column (Pharmacia) equilibrated with 0.05 M Tris-buffered saline, pH 8.0, and eluted with 0.04 M sodium Citrate, pH 4.5. Eluted fractions are immediately buffered to a pH of 7.4 by addition of one-tenth vol of 1 M Tris, pH 8.0. Fractions with greatest absorbance at 280nm are then pooled and dialyzed against PBS overnight at 4°C.

TRANSIENT EXPRESSION OF Epo/Ig IN COS I CELLS

Alternatively, COS I cells are grown to 70% confluency in Dulbecco's Modified Eagle Medium, high glucose (4.5 g/L; Gibco), 10% fetal bovine serum (Hyclone) in the presence of 100 U penicillin, 100 µg streptomycin, 250 ng Fungizone per ml of tissue culture medium (antibiotic-antimycotic cocktail from Gibco) at 37°C and 10% CO_2. The cells are harvested by trypsinizing using 0.05% Trypsin, 0.53 mM EDTA (Gibco) and washed twice with phosphate buffered saline (PBS)/6mM glucose solution. Cells are suspended in the above PBS/glucose buffer to a concentration of 2 x 10^6 cells/ml. 0.5 ml. of cells are placed in electroporation cuvettes (0.4 cm gap, Bio-Rad) and 10 µg of Epo/Ig cDNA added. The cells are electroporated under the following conditions: voltage = 0.3 kV, field strength = 0.75 kV/cm, capacitor = 250 µF, and resistor = none (Pulse controller set at Ω). Cells are plated in 30 ml of pre-warmed DMEM, high glucose, 10% FBS and incubated for 72 h at 37°C and 10% CO_2.

The conditioned media is collected and centrifuged at 13,800 x g for 10 min at 4°C. 1 ml aliquots of each conditioned media are dialyzed against Minimum Essential Medium α overnight with 3x changes of medium. These samples are assayed for EPO activity by the method of Krystal.

STABLE EXPRESSION OF Epo/Ig IN CHO CELLS

Chinese Hamster Ovary cells (CHO) and NS.1 myeloma cells can be used for stable expression of Epo/Ig protein.

CHO cells are grown to 70% confluency in Dulbecco's Modified Eagle Medium, high glucose (4.5 g/L; Gibco), 10% fetal bovine serum (Hyclone) in the presence of 100 U penicillin, 100 µg streptomycin, 250 ng Fungizone per ml of tissue culture medium (antibiotic-antimycotic cocktail from Gibco) at 37°C and 10% CO_2. The cells are harvested by trypinsizing using 0.05% Trypsin, 0.53 mM EDTA (Gibco) and washed twice with phosphate buffered saline (PBS)/6mM glucose solution. Cells are suspended in the above PBS/glucose buffer to a concentration of 2 X 10^6 cells/ml. 0.5 ml. of cells placed in electroporation cuvettes (0.4 cm gap, Bio-Rad) and 20 µg of linearized pcDNA/EPO/hinge/EPO plasmid DNA is added. The cells are electroporated under the following conditions: voltage = 1.5 kV, field strength = 0.75 kV/cm, capacitor = 3 µF, and resistor = none (Pulse controller set at Ω). Cells are plated in 30 ml. of pre-warmed DMEM, high glucose, 10% FBS containing 1.5 mg/ml of G418 (Geneticin, Gibco BRL) and incubated at 37°C and 5% CO_2. After subcloning, high producing clones are selected by screening supernatants for EPO by ELISA (PharMingen, San Diego, CA). The control is 10 µg of pcDNA-EPO.

IN VITRO CHARACTERIZATION OF Epo/Fc

Affinity-purified proteins are characterized by Laemmeli gel electrophoresis under reducing (+DTT) and nonreducing (-DTT) conditions. After transfer to a nylon membrane (Imobilon-P, Millipore, Bedford, MA) the protein is: 1) visualized by Coomassie blue staining, and 2) analyzed by Western blot to confirm the IgG2a isotype

specificity using a rat ant-mouse IgG2a as the primary Ab, a biotinylated rabbit anti-rat mAb as the secondary Ab, and visualized with avidin-HPRO complex (Vector) using 3',3'-diaminobenzidine (DAB; Vector) for detection of enzyme activity.

CONFIRMATION OF EPO ACTIVITY

Erythropoietin-activity of the EPO/Ig fusion protein is assayed as described in U.S. Patent 5,580,853, the teachings of which are hereby incorporated by reference.

Samples of fusion protein are evaluated for biological activity according to the method of Krystal, G., Exp. Hematal., 11: 649-660 (1983). Briefly, the bioassay of Krystal measures the effect of erythropoietin on intact mouse spleen cells. Mice are treated with phenylhydrazine to stimulate production of red blood cell precursor cells in the spleen. After treatment, the spleens are removed, intact spleen cells are carefully isolated and incubated with various amounts of wild type erythropoietin or the modified erythropoietin described herein. After an overnight incubation, ^{3}H thymidine is added and its incorporation into cellular DNA is measured. The amount of ^{3}H thymidine incorporation is indicative of erythropoietin-stimulated production of red blood cells via interaction of erythropoietin with its cellular receptor.

In vivo activity of the fusion proteins is tested as follows: Conditioned medium from cells transfected with mEPO/Ig is used to inject mice (B6C3F1 strain, female, Jackson Labs, 18g). The hematocrits of these mice are measured prior to administering the fusion protein. Approximately 300 U of fusion protein per kg of mouse are

injected subcutaneously on day 1, day 3 and day 5. Their hamatocrits are measured on day 7.

A group of New Zealand white rabbits are injected intravenously with EPO/Ig fusion protein ml in PBS. Blood samples are obtained at 5 minutes and 2, 4, 6, 9,and 24 hours, or at longer time periods, and activity of the circulating erythropoietin fusion protein is measured.

EQUIVALENTS

Those skilled in the art will recognize, using no more than routine experimentation, many equivalents of the invention described specifically herein. Such equivalents are intended to be encompassed by the scope of the following claims.

CLAIMS

We Claim:

1. A nucleic acid encoding a fusion protein comprising erythropoietin, or an erythropoietin-like molecule, and an immunoglobulin constant region, or a fragment, analog, variant, mutant or derivative thereof, wherein the erythropoietin is fused to the immunoglobulin constant region through the carboxyl- or amino-terminal.

2. The nucleic acid of Claim 1 wherein the encoded immunoglobulin constant region is selected from the group of immunoglobulin constant regions consisting of: IgG1, IgG2, IgG2a, IgG3, IgG4, IgM, IgA, IgD or IgE.

3. The nucleic acid of Claim 2 wherein the encoded immunoglobulin constant region comprises the constant region of the heavy chain.

4. The nucleic acid of Claim 3 wherein the encoded immunoglobulin constant region comprises immunoglobulin hinge region, CH2 domain and CH3 domain.

5. The nucleic acid of Claim 4 wherein the encoded immunoglobulin constant region lacks complement fixation activity and Fc receptor binding activity.

6. The nucleic acid of Claim 2 wherein the encoded immunoglobulin constant region comprises the constant region of the light chain.

7. The nucleic acid of Claim 6 wherein the immunoglobulin constant region comprises a CL1 domain.

8. A fusion protein comprising a polypeptide comprising two components, wherein the first component is erythropoietin, or an erythropoietin-like molecule, and the second component is an immunoglobulin constant region polypeptide, or a fragment, analog, mutant variant or derivative thereof.

9. The fusion protein of Claim 8 wherein the erythropoietin is non-glycosylated erythropoietin.

10. The fusion protein of Claim 8 wherein the erythropoietin is a mutant erythropoietin selected from the group consisting of: alanine 101 erythropoietin; alanine 103 erythropoietin; alanine 104 erythropoietin; alanine 105 erythropoietin and alanine 108 erythropoietin.

11. The fusion protein of Claim 8 wherein the erythropoietin is fused at its C-terminus to the immunoglobulin constant region polypeptide.

12. The fusion protein of Claim 8 wherein the erythropoietin is fused at its N-terminus to the immunoglobulin constant region polypeptide.

13. The fusion protein of Claim 8 wherein the immunoglobulin constant region polypeptide is selected from the group of immunoglobulin constant regions consisting of: IgG1, IgG2, IgG2a, IgG3, IgG4, IgM, IgA, IgD or IgE.

14. The fusion protein of Claim 13 wherein the immunoglobulin constant region polypeptide comprises the heavy chain of the constant region.

15. The fusion protein of Claim 14 wherein the immunoglobulin constant region polypeptide comprises immunoglobulin hinge region, CH2 domain and CH3 domain.

16. The fusion protein of Claim 15 wherein the immunoglobulin constant region polypeptide lacks complement fixation activity and Fc receptor binding activity.

17. The fusion protein of Claim 13 wherein the immunoglobulin constant region polypeptide comprises the light chain of the constant region.

18. The fusion protein of Claim 17 wherein the immunoglobulin constant region polypeptide comprises a CL1 domain.

19. The fusion protein of Claim 8 wherein two, or more, fusion proteins covalently associate to form a fusion protein multimer.

20. The fusion protein multimer of Claims 19 wherein the covalent association is by disulfide linkage.

21. A method of treating anemia in a vertebrate comprising administering to the vertebrate a fusion protein according to Claim 8 in a pharmaceutical composition.

22. A method of producing an erythropoietin/immunoglobulin fusion protein comprising;

a) inserting a nucleic acid sequence encoding the erythropoietin/immunoglobulin fusion protein into an expression vector;

b) transforming competent mammalian host cells with the expression vector of a) and maintaining the cells under culture conditions suitable for the expression of the erythropoietin/immunoglobulin fusion protein; and

c) recovering the expressed immunoglobulin from the cultured cells.

23. An expression vector comprising a nucleic acid insert encoding an erythropoietin/immunoglobulin fusion protein.

24. A host cell comprising the expression vector of Claim 23.

25. A pharmaceutical composition comprising an erythropoietin/immunoglobulin fusion protein and a pharmaceutically acceptable carrier.

26. A method of treating a hematopoietic disease or deficiency in an individual comprising administering to the individual an effective amount of an erythropoietin/immunoglobulin fusion protein.

INTERNATIONAL SEARCH REPORT

Interr. al Application No
PCT/US 98/13930

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C12N15/85 C12N5/10 C07K19/00 A61K38/18
//C12N15/12,C12N15/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 464 533 A (BEHRINGWERKE AG ;GEN HOSPITAL CORP (US)) 8 January 1992 see column 1, line 1-5 see column 1, line 39-55 see column 2, line 39-45 see example 3 see figure 8 see claim 16	1-4,6-8, 11-15, 17,18, 21-26
Y		5,9,10, 16,19,20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 October 1998	27/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/US 98/13930

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MODI N.B.: "Pharmacokinetics and pharmacodynamics of recombinant proteins and peptides" J.CONTROLL. REL., vol. 29, 1994, pages 269-281, XP002080203 see abstract see pages 271-273 "Erythropoietin" ---	1,2,8, 11-13, 21,25,26
Y	WO 97 00319 A (SMITHKLINE BEECHAM PLC ;BROWNE MICHAEL JOSEPH (GB); CHAPMAN CONRAD) 3 January 1997 see page 1, line 31-37 see page 2, line 3-9 see page 2, line 19-24 ---	5,16
Y	WO 94 02611 A (NEW ENGLAND DEACONESS HOSPITAL) 3 February 1994 see page 1, line 19-25 ---	9
Y	GRODBERG J. ET AL.: "Alanine scanning mutagenesis of human erythropoietin identifies four amino acids which are critical for biological activity" EUR.J.BIOCHEM., vol. 218, 1993, pages 597-601, XP002080204 see abstract ---	10
Y	WO 95 25746 A (NEW ENGLAND DEACONESS HOSPITAL) 28 September 1995 see page 3, line 28 - page 4, line 18 see claims ---	19,20
A	BATRA J K ET AL: "INSERTION OF CONSTANT REGION DOMAINS OF HUMAN IGG1 INTO CD4-PE40 INCREASES ITS PLASMA HALF-LIFE" MOLECULAR IMMUNOLOGY, vol. 30, no. 4, March 1993, pages 379-386, XP000676597 see page 379, right-hand column, line 13-28 see page 380, left-hand column, line 17-40 see page 386, left-hand column, line 25-31 ---	1-4,6-8, 11-15, 17,18, 23-25
A	JELKMANN W: "Biology of erythropoietin" CLIN.INVESTIG., vol. 72, 1994, pages S3-S10, XP002080205 see abstract -----	1-26

2

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US 98/ 13930

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 21, 26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. al Application No
PCT/US 98/13930

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0464533 A	08-01-1992	AT 169030 T	15-08-1998
		AU 655421 B	22-12-1994
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		EP 0751959 A	08-01-1997
		US 5747446 A	05-05-1998